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Comparison of the in vitro effect of two different corneal Crosslinking-Protocols on fungal growth

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Inhaltsverzeichnis

Abstract:

Background: Corneal Cross-linking (CXL) is a combination of irradiation with UV-A light and administration of Riboflavin as a photosensitizer. The subsequent formation of free oxygen radicals results in the formation of crosslinks at the surface of collagen fibers and within the surrounding extracellular matrix, which in turn causes biomechanical stiffening and biochemical stabilization of the cornea. Additionally, free oxygen radicals damage microorganisms and have an antimicrobial effect. For these reasons CXL is regarded as a good therapy option for infectious keratitis, including corneal ulcerations. CXL has proven efficacy against bacterial keratitis in vitro and in vivo.

Candida albicans is a possible cause of infectious keratitis that has been isolated from equine patients in the northwestern USA and from human patients world-wide. Equine keratomycoses in general are associated with extended and expensive therapy and may result in blindness or loss of the eye.

Methods: Two CXL protocols with a different total dose of UV-A energy applied (5.4J/cm^2 and 16.2J/cm^2) were tested on *Candida albicans* in vitro. The aim of this study was to evaluate a CXL energy dependent growth suppression effect on *Candida albicans* after irradiation with UV-A light in combination with the photosensitizer Riboflavin.

Results: Within our in vitro experimental design, CXL demonstrated no significant effect on *Candida albicans* growth, independent of the UV-A energy dose.

Conclusion: The two tested protocols of CXL do not seem to have a fungicidal or suppressing effect on *Candida albicans* in vitro. Even with the high-energy protocol and the reduced Riboflavin concentration to increase the penetration depth there is no reaction visible. So *Candida albicans* does not seem to be sensitive to UV-A light in combination with Riboflavin as photosensitizer within the applied study conditions. But even without a direct antifungal effect CXL may be a good option as an additional therapy to biomechanically stiffen and biochemically stabilize the remaining cornea.

Zusammenfassung:

Corneal Cross-linking (CXL) ist eine Kombination von UV-A Bestrahlung und Riboflavin, welcher als Photoaktivator wirkt. Die darauf folgende Bildung von freien Sauerstoffradikalen resultiert in einer Quervernetzung von Kollagenfasern und der umgebenden extrazellulären Matrix. Dies wiederum führt zu einer biomechanischen Festigung und biochemischer Stabilisierung der Hornhaut. Zudem können die freien Radikale Mikroorganismen schädigen, was anhand von Bakterien bereits in vitro und in vivo gezeigt werden konnte.

Candida albicans ist eine mögliche Ursache von infektiösen Keratitiden. Sie wurden bereits aus equinen Patienten im Nordwesten der USA und aus humanen Patienten weltweit isoliert.

In dieser Studie wurden zwei unterschiedliche CXL Protokolle mit unterschiedlichen totalen Energiedosen (5.4J/cm^2 und 16.2J/cm^2) an Candida albicans in vitro untersucht. Das Ziel der Studie war eine potentiell Energieabhängige Wachstumshemmung von Candida albicans nach CXL zu messen.

In unseren in vitro Experimenten konnte jedoch keinerlei hemmende Wirkung von CXL auf das Wachstum von Candida albicans festgestellt werden. Und das unabhängig von der totalen Energiedosis.

Doch trotz diesen Resultaten kann CXL eine gute Option als Zusatz-Therapie von Keratomycosen darstellen, da der Effekt biomechanischen Festigung und biochemischen Stabilisierung der Hornhaut erhalten bleibt.

Introduction:

Corneal Cross-linking (CXL) was originally developed for the treatment of keratoconus in human medicine.[1] It was first performed in the 1990s at Dresden University in Germany.[2] CXL occurs by combining UV-A irradiation with the photosensitizer Riboflavin. The irradiation with UV-A light leads to an increased corneal rigidity[3] and stiffens the anterior corneal stroma[4] by the creation of intralamellar covalent collagen bonds.[5] This effect is limited to the anterior 200µm to 300µm of the treated cornea.[4] Riboflavin, also known as vitamin B2, is an important component of the CXL process. Riboflavin acts as a photosensitizer. It absorbs UV light and reacts directly with the collagen protein. Free radicals like superoxide anion lead to hydrogen and hydroxol radicals, which can react further with collagen molecules, inducing chemical covalent bonds between collagen molecules and proteoglycans and within the proteoglycan-rich corneal stromal extracellular matrix.[6] CXL can also lead to direct damage of pathogen nucleic acids, proteins, and membranes by reactive oxygen species or damage to the genetic machinery by the interaction of Riboflavin with microbial nucleic acids.[7,8]

Furthermore, *Spoerl et al*.[9] found that cross-linked corneas had increased resistance against enzymatic digestion with pepsin and collagenase.[2,10,11] Also, clinically, CXL seems to lead to a reduction in corneal inflammation.[12]

CXL can thus rigidify and stabilize a tissue but also damage viable cell populations and represents a potential danger to important structures of the eye, including the corneal endothelium, lens epithelium and retina. Several studies demonstrated that the cytotoxic damage after CXL was restricted to the anterior 200[13] - 300µm[14-16] of the corneal stroma. Indeed, the energy level that the corneal endothelium is exposed to is less than half the endothelial damage threshold in a Riboflavin-saturated cornea of ≥400 µm thickness exposed to the standard CXL procedure.[14,17] Therefore, all structures behind a 400 µm-thick corneal stroma, including the corneal endothelium, iris, lens epithelium and retina, are exposed to a residual UV radiation exposure that is regarded as safe for these structures. [17,18] These statements apply to a normal human cornea saturated with Riboflavin and treated with the conventional CXL protocol, the so-called Dresden protocol. This protocol consists of a 30 minute pretreatment with 0.1% Riboflavin drops until Riboflavin starts appearing in the anterior chamber of the eye, which implies complete saturation of the corneal stroma with Riboflavin. Subsequently, the cornea is irradiated for 30 minutes with UV-A at a wavelength of 365 nm and a power density of 3mW/cm² (total fluency: 5.4J/cm²). As stated above, the safety values are calculated for the normal human cornea, the thickness of which is similar to the thickness of the normal feline and canine cornea. Horses however have a thicker cornea (812.0 ± 44.1 µm)[19] than humans (548.16±48.68 µm)[20], cats (629.08 ± 47.05 µm)[21] and dogs (611.2 ± 40.3 µm)[22]. If the CXL effect would be restricted to the anterior 200[13] - 300µm[14-16] of the normal equine cornea of 812.0± 44.1 µm thickness, the proportional treatment effect would be much more superficial in the horse compared to humans, dogs or cats. This means that the safety values could be recalculated to arrive at a deeper treatment effect. In a cornea which is +/- 640µm thick (estimate based on total corneal thickness – epithelial

thickness measured by OCT in horses)[19] the total fluency could be increased to 8.6J/cm² without increasing the energy level that the corneal endothelium is exposed to. This total fluency would be reached for example with a power density of 9mW/cm² for 16 minutes. The Riboflavin concentration has to be adjusted to maintain the energy level absorbed by the endothelium at 0.18J/cm². With this calculation one could obtain security measures similar to those with the Dresden protocol in the normal human cornea. (S. King, personal communication)

CXL also has a very welcome antimicrobial effect[12] which is thought to be a direct result of the UV-A radiation[23,24] and the incurred oxygen radicals[25]. In the 1960s a Japanese research group demonstrated that Riboflavin and UV-A light irradiation in combination can be used to inactivate the RNA containing tobacco mosaic virus.[26] Riboflavin interacts with the DNA or RNA in pathogens. It oxidizes nucleic acids through electron transfer reactions[27] preventing replication of the pathogen genome.[28] The combination of UV-A light and Riboflavin results also in reactive oxygen species, which can damage pathogens.[28]

In 2000 *Schnitzler et al.*[29] demonstrated CXL as an effective treatment for melting corneal ulcers, which was confirmed by others.[30,31]

A possible and potentially devastating complication of corneal ulcers is a progressive stromal degradation, so called keratomalacia or melting process. This occurs when there is an imbalance between proteases and protease-inhibitors. Clinically the corneal stroma appears gelatinous, therefore the name “melting ulcer”. In addition to broad-spectrum antibiotics and atropine administration the impact of collagenase and other proteases should be reduced. Therefore protease-inhibitors including autologous serum can be used. These compounds should be administrated every one to two hours until healing begins.[32]

Melting corneal ulcers can be caused by bacteria, fungi and protozoa.[33-36] The incidence of fungal keratitis in dogs and cats is low. Fungi, which were associated with keratitis in dogs in the literature are *Alternaria, Acremonium, Cephalosporium, Candida, Curvularia, Hormographiella, Pseuallescheria,* and *Scedosporium* spp.[37-42] Horses, however, have a much higher incidence of fungal keratitis. The equine eye is predisposed to fungal infections due to its exposed position, suspected tear film instability, and the prevalence of fungal organisms in the horse’s environment.[43] Generally the whole process starts with an epithelial defect, which allows the microorganisms to reach the stroma. Fungi have a tropism to glycosaminoglycans, which is why they are commonly found at or in Descemet’s membrane. Secondary anterior uveitis is caused by released proteases.[43]

In healthy horses in Switzerland *Alternaria, Eurotium, Rhizopum, Cladosporium, Aspergillus* spp*., Penicillium* spp*.* and *Candida guilliermondii* could be isolated from conjunctival cytobrush samples.[44,45] *Aspergillus sp*. was identified as cause of keratomycosis in horses in two studies from Switzerland and the UK. [43,46] In other studies conducted in the USA Aspergillus[47,48] and Fusarium were identified as the most common fungi causing keratomycosis in horses.[49-54] *Candida* was also detected as a cause for ulcerative keratitis in the USA.[52,53,55] Corneal ulceration is also a problem in human medicine and an important cause of blindness.[56] *Candida* is one of

the main and emerging causes of fungal keratitis in humans in certain areas of the world.[57,58]

USA	Keratomycosis	Healthy horses (samples taken from the conjunctival fornix)
Absidia		(1)[59]
Acremonium		(8)[60], (24)[45]
Actinomyces	(1)[61]	
Alternaria	(4)[61], (1)[62]	(6)[45],(12)[59], (3)[60]
Aspergillus	(7)[54](2)[61], (9)[47],(28)[48], (8)[49], (9)[50], (13)[51], (47)[53], (3)[55], (11)[62], (3)[63], (10)[64], (4)[65], (3)[66]	(8)[60], (2)[59], (20)*[67]
Botrytis		(1)[45]
Candida	(8)[53,68](1)[55], (1)[61], (2)[48], (2)[65], (?)[68]	(1)[45], (3)[60], (2)[59]
Cephalosprium		(2)[59]
Chrysosporium	(1)[54]	(9)[60]
Cladosporium	(1)[63]	(6)[45],(8)[67], (13)[59], (17)[60]
Colleotrichum	(?)[68]	
Cryptococcus	(1)[48], (2)[62]	
Curvularia	(1)[54], (1)[63], (1)[66]	(3)[60]
Cunninghamella		(2)[59]
Cylindrocarpon	(1)[50]	
Drechslera	(1)[62]	
Epidermophyton		(1)[59]
Fusarium	(7)[53](1)[61],(3)[48], (6)[49], (7)[50], (10)[51], (6)[54], (2)[62], (5)[63], (1)[65], (?)[68], (1)[69]	(1)[60], (3)[59]
Geotrichum		(2)[45]
Gliocladium		(5)[45], (1)[59]
Graphium	(1)[48]	
Helminthosporium		(1)[45]
Ibrula		(1)[45]
Microsporum	(2)[53]	
Memoniella		(1)[45]
Mucor	(2)[61], (2)[53], (2)[62], (?)[65]	
Paecilomyces	(1)[48], (2)[62], (?)[68]	(5)[60]
Papulospora	(2)[48]	
Penicillium	(2)[50], (1)[54], (1)[61],(4)[62], (1)[63]	(19)[45], (10)[60], (3)[59]
Philophora	(?)[68]	(2)[60]
Pichia	(1)[53]	
Pseudoallescheria	(1)[53],(1)[70]	
Rhizoctonia	(1)[62]	
Rhizopus	(3)[48]	(8)[60]
Rhodotorula	(1)[62]	
Saccharomyces	(1)[62]	(3)[59]

Scedosprium	(2)[48], (1)[53]	
Scopulariopsis	(1)[54]	
Scytalidium	(1)[48], (1)[50]	
Stemphyllium	(1)[53]	
Streptomyces	(2)[62]	
Torulopsis	(1)[50]	
Trichoderma	(1)[62]	(2)[45]
Trichosporon	(1)[53], (1)[65]	(2)[60]
Unidentified yeast	(1)[50], (2)[53]	(13)[60], (11)[45], (2)[59]
Verticillium	(1)[53]	(1)[60], (2)[45]

Table 1: Incidence of fungal strains isolated from equine eyes in the United States of America. The left column lists samples taken from horses with keratomycosis, the right column lists samples taken from healthy horses. () = Number of cases. * = Samples were taken from the conjunctiva and the cornea.

Europe	Keratomycosis	Healthy horses(samples taken from the conjunctival fornix)
Absidia		UK(2)[71]
Alternaria		CH(46)[44], I(1)[72]
Aspergillus	CH(2)[43], UK(2)[46] E(4)[73]	CH(14)[44], UK(3)[71], I(33)[72]
Candida		CH(2)[44], I(3)[72]
Cladosporium		CH(14)[44], I(7)[72]
Cryptococcus		I(1)[72]
Curvularia		CH(2)[44]
Deratomces		CH(2)[44]
Drechslera		I(1)[72]
Eurotium		CH(17)[44]
Fusarium	E(1)[73]	I(1)[72]
Histoplasma	CH(1)[43]	
Microsporum	E(1)[73]	
Mucor		CH(5)[44]UK(4)[71], I(7)[72]
Paecylomyces		I(1)[72]
Penicillium	E(1)[73]	CH(16)[44]
Rhizopus		CH(14)[44]
Scopulariopsis		CH(3)[44]
Trichoderma		I(1)[72]
Unidentified yeast	E(1)[73]	

Table 2: Incidence of fungal strains isolated from equine eyes in Europe. The left column lists samples taken from horses with keratomycosis, the right column lists samples taken from healthy horses. () = Number of cases, CH = Switzerland, E = Spain, I = Italy, UK = United Kingdom.

Others	Keratomycosis	Healthy horses(samples taken from the conjunctival fornix)
Acremonium		BR(2)[74]
Aspergillus	J(5)[75]	BR(62)[74], BR(23)[76]
Aureobasidium		BR(1)[74]
Bipolaris		BR(1)[74]
Candida		BR(3)[74]
Chrysosporium		BR(1)[74]
Cladorrhinum	AUS(1)[77]	
Cladosporium		BR(1)[74], BR(9)[76]
Curvularia		BR(5)[74]
Eurotium		BR(2)[76]
Fusarium	J(1)[75]	BR(3)[74], BR(1)[76]
Geotrichum		BR(1)[76]
Gliomastix		BR(1)[76]
Microsporum		BR(6)[74]
Mortierella	J(2)[75], J(1)[78]	
Mucor		BR(5)[76]
Penicillium		BR(6)[74], BR(21)[76]
Rhizopus		BR(2)[76]
Rhodotorula		BR(1)[74]
Scopluariopsis		BR(1)[74] ,BR(16)[76]
Staphylotrichum		BR(1)[76]
Syncephalastrum		BR(3)[76]
Trichoderma		BR(18)[76]
Unidentified Yeast		BR(10)[76]
Verticillium		BR(1)[76]

Table 3: Incidence of fungal strains isolated from equine eyes in Australia (AUS), Brazil (BR) and Japan (J). The left column lists samples taken from horses with keratomycosis, the right column lists samples taken from healthy horses. () = Number of cases.

Unfortunately it has been estimated that only 50% of infected eyes with melting ulcers in humans will heal with a favorable outcome for vision when diagnosis and initiation of appropriate antimicrobial treatment are delayed.[79] One of the major problems is the antimicrobial drug resistance of pathogens.[80-83] CXL shows great promise as an additional therapy for melting ulcers[30,80,84], most of all for cases which are not responding to antimicrobial therapy.[1,85,86]

Few drugs are available for topical antifungal therapy on the eye. The main problems with these drugs are the emergence of resistant strains and the poor corneal penetration.[87] This drug resistance is a result of chromosomal mutation, inductive expression of a latent chromosomal gene, or exchange of genetic material via transformation, bacteriophage transduction, or plasmid conjugation.[88,89] Antifungal agents have been evaluated in several in vivo and in vitro studies.[44,49,50,53,59] The goal is to find a drug, which the isolated fungus is susceptible for. Whether antifungal

susceptibility patterns vary between geographic locations is under debate. *Coad et al.* and *Pearce et al.* reported considerable variation in fungal sensitivities among different fungal species[62], but not among different locations.[49] Susceptibility test results from Florida, Missouri, Tennessee and Georgia were compared in the study from *Pearce et al.*[49]. Another study[53] from the northeastern United States showed different results. In contrast to the other studies from the US, this group isolated *Candida albicans* from equine patients with keratomycosis, and demonstrated low susceptibility for Miconazole.[53] An influence of geographic differences on the incidence and susceptibility of various fungal isolates was mentioned by *Forster et al.* as well.[90]

In vitro susceptibility: in this schedule the antimycotic medications are listed from most to least effective. In some studies a ranking of medications is the only information provided.
>> = significantly different susceptibility, > = different susceptibility, but no proven significance, () = percentage susceptible.

- **Aspergillus**:
    - Voriconazole > Miconazole > Itraconazole > Natamycin > Fluconazole > Ketoconazole[49]
        - Voriconazole >> Natamycin, Fluconazole, Ketoconazole[49]
    - Voriconazole > Amphotericin B > Itraconazole > Miconazole = Fluconazole[44]
    - Nystatin (96%) > Clotrimazole (91%) > Itraconazole (86%) > Natamycin (82%)>> Miconazole (30%) >> Fluconazole (8%)[53]
    - Natamycin (100%) = Miconazole (100%) = Itraconazole (100%) > Ketoconazole (78%) >> Fluconazole (0%)[50](MIC)
    - Miconazole (100%) > Nystatin (43%)[59]
- **Eurotium amstelodami:**
    - Voriconazole > Itraconazole > Amphotericin B > Miconazole > Fluconazole[44]
- **Fusarium**:
    - Natamycin > Voriconazole > Miconazole > Fluconazole > Itraconazole > Ketoconazole[49]
        - Natamycin >> Itraconazole, Ketoconazole[49]
    - Natamycin (100%) > Amphotericin B (42%) = Clotrimazole (42%) = Ketoconazole (42%) = Nystatin (42%)[53]
        - Natamycin >> Miconazole, Fluconazole, Fluorocytocin, Itraconazole[53]
    - Natamycin (71%) > Miconazole (43%) > Ketoconazole (14%) >> Itraconazole (0%) = Fluconazole (0%)[50] (MIC)
    - Miconazole (100%) > Nystatin (43%)[59]
- **Candida**:
    - Ketoconazole (100%) = Natamycin (100%) = Nystatin (100%)[53]

- **Independent of type of fungus:**
    - Nystatin (88%) = Natamycin (88%) > Clotrimazole (81%) > Itraconazole (69%) >> Miconazole (28%) > Fluconazole (16%)[53]
    - Natamycin (97%) > Nystatin (74%) > Miconazole(69%) > Amphotericin (51%) > 5-Fluorocytosine (49%) > Ketaconazole (31%) > Griseofulvin (3%)[59]
    - Natamycin = Miconazole > Itraconazole > Ketoconazole >> Fluconazole[50]

	Aspergillus	Candida	Cylindracarpon	Eurotium amst.	Fusarium	Mucor	Penicillium	Scytalidium	Trichosporon	Torulopsis	Independent of fungi
Amphotericin B	(+/-°)[44]			(+°)[44]	(+*)[91] (42%)[53]						(51%)[59]
Clotrimazol	(91%)[53]	(+*)[92]			(42%)[53]						(81%)[53]
Bifonazol		(+*)[92]									
Itraconazole	(+*)[65] (-°)[44] (86%)[53] (100%)[50]	(+*)[65]	(-°)[50]	(+°)[44]	(-*°)[65] (+*)[91] (0%)[50]	(+°)[65]	(+°)[50]	(+°)[50]	(+*)[65]	(+°)[50]	(69%)[53]
Fluconazole	(- bzw. +/-°)[44] (0%)[50] (8%)[53]	(+*)[93]	(-°)[50]	(-°)[44]	(0%)[50]		(-°)[50]	(-°)[50]		(+°)[50]	(16%)[53]
Fluorocytosin											(49%)[53]
Griseofulvin											(3%)[59]
Ketoconazole	(78%)[50]	(100%)[53]	(-°)[50]		(14%)[50] (42%)[53]		(+°)[50]	(-°)[50]		(+°)[50]	(31%)[59]
Miconazole	(+/-°)[65] (-°)[44] (30%)[53] (100%)[50] (100%)[59]		(-°)[50]	(-°)[44]	(43%)[50] (100%)[59]		(+°)[50]	(+°)[50]		(+°)[50]	(28%)[53] (69%)[59]
Natamycin	(+*)[66], (+°)[65] (82%)[53] (100%)[50]	(+*)[94] (-*)[95] (100%)[53]	(+°)[50]		(+°, -°)[54] (+*)[96] (71%)[50] (100%)[53]		(+°)[50]	(+°)[50]		(+°)[50]	(88%)[53] (97%)[59]
Nystatin	(43%°)[59] (96%)[53]	(100%)[53]			(42%)[53] (43%°)[59]						(74%)[59] (88%)[53]
Sulfadiazine	(+°)[54]				(+°)[54]						

Voriconazole	(+°)[44]			(+°)[44]	(+*)[91]						

Table 4: Reported susceptibility of fungal species to selected antifungal agents.
* = in vivo (antimycotic effect on a corneal infection in a living animal); ° = in vitro (susceptibility testing in the lab); green = horse; black = rabbit; (+) = susceptible or favorable clinical result in *in vivo* study; (-) = resistant or poor clinical result in *in vivo* study; (+/-) = intermediate susceptibility; numbers in brackets = percentage susceptible in vitro

According to this table, Natamycin seems to have a very good antifungal effect clinically as well as in vitro. Positive effects were observed in all fungi tested in the studies mentioned above. Nystatin, Miconazole, Clotrimazol and Itraconazle show relatively good effectivity too. But with these drugs the results seem to depend more on the type of fungus treated. Fluconazole shows almost no antifungal effects, independent of *in vitro* or *in vivo testing*.
Cylindracarpon seems to be a more resistant fungus than others. *Cylindracarpon* was resistant to Itraconazole, Fluconazole, Ketoconazole, Miconazole but not Natamycin. *Candida* was susceptible to all drugs tested in the studies mentioned above.

Various studies investigate the effect of CXL on fungi. The results are variable. Some of these studies demonstrated that CXL is a good adjunctive treatment for fungal keratitis.[84,86,97] *Martins et al*.[56] and *Kashiwabuchi et al.*[87], on the other hand, were not able to inhibit the growth of *Candida albicans* with CXL in vitro.[56]
The hypothesis of this study was that CXL has an energy-dependent effect on the growth of *Candida albicans*.
This experimental trial was performed to identify new strategies to kill fungi in keratomycotic patients. Therefore two different CXL protocols with a different total energy delivery were tested on *Candida albicans in vitro*. In the present study we compared the antifungal effect of two different CXL protocols on *Candida albicans*. In the first protocol the fungal colonies were irradiated with UV-A light (365 nm) at a power density of 3mW/cm² for 30 minutes. A total energy dose of 5.4J/cm² was applied. The second protocol delivers a threefold total energy dose. Here a power density of 9mW/cm^2 was used for 30 minutes. This results in a total energy dose of 16.2J/cm^2.

This high-dose protocol may be a good option to treat horses with keratomycosis because of the thicker cornea, tolerating a higher total energy dose. With a higher energy dose and decreased Riboflavin concentration the depth of CXL penetration increases, which in thick equine corneas may translate to reaching pathogens in deeper layers of the cornea without increasing the risk.

But not just the used energy has an influence on the penetration depth. The penetration depth seems to be also dependent on the species. *Gallhoefer et al.*[98] showed that CXL has an unexpectedly shallow effect in the equine cornea (median 173 µm), compared to rabbit corneas (median 274 µm). These results led us to question the clinical efficacy of the routine Dresden CXL protocol in species like horses[98]

All but one of the available studies in which the efficacy of CXL against *Candida albicans* was tested *in vitro* yielded negative results [56,87,99,100]. *Martins et al.*[56] inoculated Mueller-Hinton agar plates with a cotton swab and performed a disc diffusion susceptibility test where they measured the growth inhibition zone (GIZ) after irradiation and a 24h incubation period. A Riboflavin concentration of 0.1% was used, as in all other studies under similar conditions.. Drops of Riboflavin were placed directly adjacent to the discs. They let the drops diffuse in the agar media for 20 minutes and started the UV-A irradiation (3mW/cm^2) for one hour. This experiment was performed three times for each microorganism (susceptible and resistant strains of different bacteria and Candida albicans).[56] *Sauer et al*.[100] tested the in vitro effect of CXL on fungal growth using a disc diffusion susceptibility assay. In a second step amphotericin B tablets were added to the discs as pretreatment. Apart from amphotericin B pretreatment the experimental design was similar to the one of *Martins et al*.[56]. Antifungal pretreatment in combination with CXL inhibited growth of C. albicans growth, CXL alone however did not.

Kashiwabuchi et al.[87] performed a similar experiment in 96 well plates (diameter 6.85mm) filled with 100µl of sabouraud dextrose agar. A 10µl aliquot of a single inoculum was added and incubated for 72 hours. They added 40µl of 0.1% Riboflavin to each well and then irradiated the wells (3mW/cm^2) for 30 minutes. After the irradiation the Riboflavin was drained, 100µl of saline was added to each well and the cells were recovered. Then the samples were inoculated onto a sabouraud dextrose agar plate and incubated at 25°C for 48h. Cell viability was measured employing the trypan blue exclusion method and the number of colony forming units per millimeter (CFU/ml) was calculated.[87]

The only study yielding positive results was from *Bing sun et al*.[99], on which our study design was based. Several dilutions were produced to reach cell concentrations at 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/ml. Samples were incubated in culture tubes and shaken in the dark at room temperature for 30 minutes. 200µl aliquots of the suspensions were pipetted into single wells on a 48 well plate with a diameter of 11mm. Exposure to UV-A illumination was conducted in a darkened environment to prevent photosensitization and subsequent loss of Riboflavin to background light. The wells were irradiated for 30 minutes (3mW/cm^2). The suspensions were then serially diluted 10 fold with saline and 100µl aliquots of the dilutions were seeded in

duplicate onto sabouraud glucose agar. The agar plates were incubated for 48 hours at 25°C, colonies were counted and cell concentrations were calculated.[99]
Two energy delivery protocols were evaluated in our study, with the aim to evaluate an energy-dependent inhibition of fungal growth. The higher-energy dose protocol also may be a promising treatment option for horses, because equine corneas are thicker than small animal and human corneas. A higher energy delivery might enable clinicians to more effectively combat microorganisms and to penetrate deeper into the thick corneas of horses.

Background:

Anatomy and physiology of the cornea:

The cornea is avascular and acts as the primary refractive element and structural barrier of the eye. The cornea consists of 4 layers: epithelium, stroma, Descemet's membrane and the endothelium. The epithelium is covered by the tear film, which nourishes and protects the cornea. The tear film also supplies immunological and growth factors that are important for epithelial health, proliferation and repair.[101] The epithelium consists of a single layer of cuboidal basal cells, followed by several layers of so called wing cells. The outermost layer is formed by squamous cells, which are not keratinized. The lifespan of corneal epithelial cells is about 7 to 10 days, which results in a complete turnover of the corneal epithelial layer every week. Tight junctions connect the surface cells. This and the overall lipophilic nature of the epithelium prevents fluids from the tear film to enter the cornea. This may be visualized by means of the fluorescein dye test. When the epithelium is intact, fluorescein cannot enter into the cornea and the cornea does not retain the stain. However, when there is an epithelial defect, fluorescein can enter the corneal stroma and the injured spot of the cornea will reflect a green color, especially when exposed to blue light.[102] The close connection between the cells also represents the major barrier to drug diffusion and pathogen penetration. The deepest cellular layer of the corneal epithelium is the basal cell layer. Basal cells build a single cell layer and are attached by hemidesmosomes and anchoring fibrils to the underlying basement membrane. This basement membrane is about 0.05µm thick and contains type IV collagen and laminin. The basal cells are the main proliferating cells and the reason for epithelial cell turnover. Bowman's layer lies between the corneal epithelium and the stroma. This is not a true membrane but an adaptation of the anterior stroma which helps the cornea to maintain its shape.[102] Bowman's layer has not been consistently identified in animals.

The stroma represents about 80% to 85% of the corneal thickness. An important characteristic is the precise organization of the stromal fibers and extracellular matrix. This parallel layering of lamellae and the shift in collagen fiber orientation between lamellae are the main reasons for corneal strength and mechanical barrier function. The precisely regulated and small diameter of (+/- 30 nm) and distance between (+/- 40 nm) the collagen fibers are the main reasons for corneal transparency. The predominating cells in the stroma are the keratocytes, which are mesenchymal cells responsible for the production, deposition, organization and remodeling of the extracellular matrix[102], which consists primarily of collagen type I with lesser amounts of collagen type V and proteoglycans.[103]

Descemet's membrane is located between the posterior aspect of the corneal stroma and the underlying endothelium.[102]

The endothelium is a monolayer, which builds the innermost layer of the cornea. Lateral interdigitations and gap and tight junctions are present between endothelial cells. Na+, K+-ATPase pumps are abundant. These are much needed to maintain the relatively dehydrated state of the cornea, another important factor which keeps the cornea clear and transparent.[102]

Corneal injuries and healing:

Most injuries of the epithelium are mechanical in nature and entire cell layers are generally lost, leaving a defect in the epithelial layer. As a reaction, the epithelial cells begin to cover the defect within minutes by a combination of cell migration and cell spreading.[102] This early non mitotic wound coverage phase can proceed at a rate of 60 to 80µm per hours.[104] Twenty-four to 30 hours after the injury the mitotic wound healing phase starts. In this phase the epithelial cell population begins to restore numbers.[102]

When the stroma is injured the healing process resembles the healing process of the skin. It consists of three stages: repair, regeneration and remodeling.[105] First, keratocytes directly adjacent to the wound site undergo apoptosis. Keratocytes surrounding this area are then activated and migrate to the stromal injury. Activated fibroblasts and myofibroblasts are involved in the stromal remodeling after one to two weeks. The increased expression of MMPs (proteolytic enzymes) lead to extracellular matrix remodeling, cell-matrix interaction, inflammatory cell recruitment and cytokine activation. When penetrating injuries heal by excessive deposition of fibrotic repair tissue, leading to scarring and contracture, the normal function can be inhibited. The clarity and shape of the cornea will be affected.[105]

Endothelial damage is mostly combined with penetrating injuries. The damaged cells are replaced by enlargement of the surrounding cells and their centripetal migration into the injured region.[102]

Proteinases, growth factors, and cytokines produced by epithelial cells, stromal keratocytes, inflammatory cells, and the lacrimal glands are involved in the corneal healing process.

During the normal low turnover and remodeling of the corneal stroma proteolytic enzymes perform a physiological function. Important is a balance between these proteases and protease inhibitors. As long as this balance is maintained the eye stays healthy. But as soon as the proteases outweigh their inhibitors, an increased degradation of the corneal stromal collagen, proteoglycans and other components of the stromal extracellular matrix follows. This is called keratomalacia or corneal melting.[32]

Corneal ulcers:

Clinical signs for corneal ulcerations include lacrimation, blepharospasm, photophobia, conjunctival hyperemia, corneal edema, positive fluorescein staining, corneal neovascularization, inflammatory cell infiltration into and potentially loss of stromal tissue, leading to ulcer deepening. A secondary uveitis can occur due to the corneal ulcer. This manifests as miosis, iris hyperemia, increased protein levels (aqueous flare) and the presence of cells or even a hypopyon in the aqueous humor.[32]

In general, corneal defects can be caused by trauma, preocular tear film deficiencies, irritants, eyelid or eyelash abnormalities, immune-mediated or allergic inflammation, foreign bodies or the inability to blink. In dogs and cats additional risk factors are

breed related brachycephalic craniofacial and eyelid conformation. [106] It is important to identify the cause of the corneal ulcer and attempt to eliminate it, if possible.[32] Bacteria, fungi or more rarely protozoa can infect corneal defects. When infected, these defects can develop into complicated and severe ulcers, in contrast to superficial defects/erosions, which usually heal rapidly. When an infection is suspected samples for microbiologic culture and antibiotic susceptibility testing and a cytologic examination should be collected.[32]

Corneal ulcers can be classified by the depth of corneal involvement and by their underlying cause. The classifications, which consider the depth of the ulcer include superficial corneal erosions/ulcers (epithelial defects), stromal corneal ulcers, descemetoceles, and perforations. Epithelial defects normally heal within 2-6 days. The eye should be treated with ophthalmic antibiotic preparations, for example oxytetracycline drops in cats, or gel-type tear replacement if truly no infection is present. This should be applied three to four times a day to prevent secondary bacterial infection. Additionally a mydriatic agent like atropine should be given to control a corneal axonal reflex-induced ciliary muscle spasm. When the corneal stroma is involved, as evidenced by the presence of inflammatory cell infiltrates or the loss of stromal tissue, a sample should be cultured in every case due to the high likelihood of microbial infection. Any visible defect/depression in the corneal surface suggests stromal tissue loss, because most ulcers involving only the epithelium are not readily visible and require fluorescein staining for a definitive diagnosis. If a rapid stromal loss or melting is present, intensive topical antibiotic and anticollagenolytic therapy is indicated. The goal is to normalize the proteolytic activity in the tear film. The use of protease inhibitors is therefore recommended, for example autologous serum, N-acetyl-cysteine or disodium ethylenediaminetetraacetic acid. They should be instilled in the eye every 1-2 hours until healing begins. A bactericidal antibiotic should be applied every one to two hours. Surgical procedures like conjunctival grafting, collagen patches (ACell, BioSIS), amniotic membrane placement or CXL are indicated in distinct ulcers. Conjunctival grafts for example are most commonly used for chronic, infected, or progressive corneal ulcers. They provide corneal support, fibrovascular tissue to fill the corneal defects and bring a blood supply to the lesion. Amniotic membranes on the other hand have been described for treatment of corneas after superficial keratectomy and bullous keratopathy and for corneal reconstruction after removal of an inclusion cyst and dermoids. [32]

A descemetocele is an ulcer in which a full-thickness loss of corneal stroma has occurred and Descemet's membrane has been exposed. These ulcers can easily rupture and are therefore handled as emergencies. A corneal perforation occurs as soon as Descemet's membrane ruptures. A secondary iris prolapse may occur. These two situations may have a poor prognosis for saving the eye as well as for vision when the lesion is extensive. But it depends much of the dimension of the corneal perforation. Also the breed plays an essential role. Most small descemetoceles and perforations can be repaired successfully using conjunctival grafts, corneoscleral or corneoconjunctival transpositions or corneal transplantations.[32]

As mentioned above corneal ulcers can be caused by bacterial, viral and fungal infections. Bacterial keratitis is the most common type of corneal infection in dogs. Staphylococcus spp., Streptococcus spp. and P. aeruginosa are most frequently involved. Viral keratitis is mostly caused by feline and canine herpesvirus-1 (CHV-1) in cats and dogs, respectively[32] ,and rarely by equine herpesvirus-2 & 5 (EHV-2 & 5) in horses[107]. Typically these ulcers remain superficial unless complicated by secondary bacterial infections. These epithelial defects should be treated with topical antibiotics, atropine and antiviral therapy.[32]
A keratomycosis is a corneal ulcer infected by fungi. This is relatively common in horse, as mentioned above. In dogs it is considered rare. The clinical appearance of keratomycoses can be divided in three different types: the superficial punctate keratitis, stromal ulcerative keratitis with fungal plaques or corneal furrows and stromal abscess. The results in keratomycosis therapy are different. *Ball et al.*[65] for example were successful in eight of ten eyes (80%) which completely resolved after itraconazole-dimethyl sulfoxide therapy. In the study of *Voelter et al.*[43] 23 out of 36 (63.9%) eyes could be saved. But in this study surgeries like lamellar keratectomies and combinations of lamellar keratectomies and a conjunctival flap were used in addition to medical treatment. Unfortunately some of these cases resulted in corneal perforation, which can necessitate enucleation of the globe.[43] In a follow-up study of *Andrew et al.*[51] the visual outcome was favorable in 92.3% of the eyes

Materials & Methods:

Fungal strains:

For this study two different isolates of *Candida albicans* were selected which were isolated from human patients of the CHUV (Centre Hospitalier Universitaire Vaudois). The isolates were cultivated on sabouraud plates and were passaged on a new sabouraud plate every two weeks. The plates were then incubated at 30°C for 48 hours and were stored at 4.0°C afterwards.

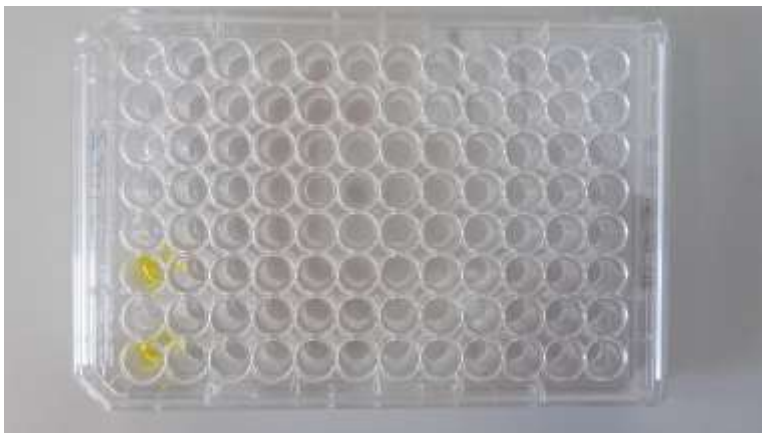
In-vitro assays:

Single loops of the Candida strains were agitated in 8ml sabouraud liquid in the morning (9.00 am) and cultivated for one day (7 hours) at 30°C while shaken at 200 rpm. In the evening (16.00 pm) the optical density (OD) of the suspension was measured and that suspension was further diluted to reach an optical density of 1.0. The optical density was measured with a biophotometer (Eppendorf, Dr. Vaudaux AG). This suspension was used to make five different dilutions: 1:2'000, 1:1'000, 1:500, 1:250 and 1:125. These dilutions were prepared as follows: Every vessel was filled with 4ml sabouraud liquid. 2µl of cultivated suspension was added to the first vessel (1:2'000), 4µl to the second (1:1'000), 8µl to the third (1:500), 16µl to the fourth (1:250) and 32µl to the fifth (1:125). At 17.00 pm these dilutions were placed in the incubator again for cultivation overnight (30°C, shaken at 200 rpm).
The next morning (8.00 am) the OD of these five fluids was measured. The goal was to have an OD as close to 1.0 as possible. ODs between 0.900 and 1.100 were considered acceptable for use in the experiments. The other dilutions were kept at room temperature so that the OD was still rising, but not too fast.
Serial dilutions were made as follows with the suspension with acceptable OD, to reach a maximal dilution of 10^{-5}. 800µl of cultivated suspension was added to the first tube. 720µl sabouraud liquid was added to the second, third, fourth and fifth vessel. An aliquot of 80µl was taken from the first tube and added to the second tube. This vessel was then shaken well and an aliquot of 80µl was taken from the second vessel and added to the third vessel, and so on.
The dilution in the fifth tube was then divided into two equal parts, resulting in two tubes with 400µl diluted Candida suspension each. 35µl of sabouraud liquid was added to one of these tubes and 35µl Riboflavin (Vitamin B2 Streuli Inj Lös 10 mg/2ml) to the other to reach a riboflavin concentration of 0.5%. The two tubes were then shaken again on a vortex mixer.
The next step was to put a 30µl aliquot of Candida suspension in sabouraud (from the first tube) in two different wells of a sterile 96 well with flat bottom tissue culture plate and repeat with a 30µl aliquot of Candida suspension + Riboflavin in sabouraud (from the second tube). The wells have a diameter of 6.6mm. A second 96 well plate

was prepared identically. Then aluminum wrapped around the plates was used to protect the Riboflavin from ambient light.
The following treatment and control groups were included on every plate:

- Control (C = no irradiation, no Riboflavin): C3mV, C9mV.
- UV only (UV = UV-A irradiation, no Riboflavin): UV3mV, UV9mV.
- Riboflavin only (R = no irradiation, with Riboflavin): R3mV, R9mV.
- UV + Riboflavin (UV +R = UV-A irradiation and Riboflavin): UV + R3mV, UV + R9mV.

On one plate the suspensions were exposed to a total energy dose of 5.4J/cm² of UV-A light (365 nm) at a power density of 3mW/cm² for 30 minutes. On the second plate a threefold total energy dose was applied. Here a power density of 9mW/cm^2 was used for 30 minutes, resulting in a total energy dose of 16.2J/cm^2.
The plates were wrapped in aluminum foil, exposing only the well exposed to the UV-A irradiation at a given time. A paper sheet was placed over the UV lamp to optimally shield the suspension from ambient room light. The two plates were always irradiated in parallel with the two lamps (9mV/cm^2 and 3mV/cm^2).
After one 30-minute irradiation period the plates were vortex shaken again to resuspend the fungi as well as possible. The aluminum foil was then reapplied and a second well left exposed to be irradiated.
The wells were irradiated in random order of appearance. When Riboflavin containing wells were irradiated first a 30-minute Riboflavin saturation lag period between aliquotting and irradiation was observed. The plates were kept wrapped in aluminum foil for 30 minutes at room temperature before irradiation during these experiments.
After the irradiation period the plate was shaken again on the vortex mixer and the suspensions in the wells were aspirated. Every well was rinsed with 40µl sabouraud fluid and all aspirated fluid was placed on a sabouraud plate. The contents of every well were placed on separate sabouraud plates, spread out and the plates were incubated for 24 hours (± 6h) at 30°C.
After the incubation period the plates were taken out and the colonies were counted (Colony counter, SC6, Stuart). Counting was performed only once a day, which explains why not all plates were incubated for exactly 24 hours. However, the eight plates composing one trial were always incubated in parallel. The duration of incubation only varied between trials.
The experiment was repeated 14 times with each lamp and each isolate.



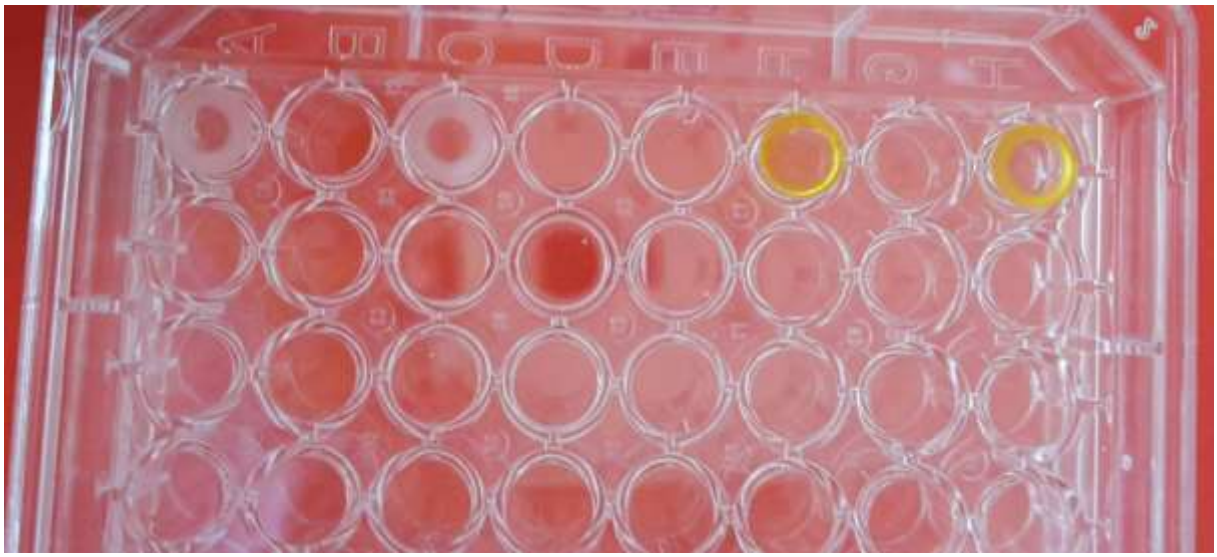
Picture 1: 96 well plate with 30 µl aliquots of *Candida albicans* and sabouraud solution suspension in wells A1 and C1, and 30µl aliquots of *Candida albicans*, LB and Riboflavin suspension in wells F1 and H1

Picture 2: 96 well plate wrapped in aluminum foil to prevent Riboflavin depletion caused by ambient light and UV-A light

In a pilot test phase several experiments were performed before the real trial started. Several pilot tests with 0.1% Riboflavin were performed with the low energy protocol and once with the high energy protocol. These experiments were performed with all of the five different concentrations of the serial dilution (picture 3). We focused on the lowest dilution because single colonies could only be counted with this dilution.

Picture 3: Five different dilutions (serial dilution) were irradiated and spread out on sabouraud agar plates and incubated for about 24h.

Incubation after irradiation in the wells of the 96-well plate (where the solution was irradiated) instead of on the sabouraud plates was also attempted several times. Optical density measurement with the tecan reader (Tecan ELISA-Mikrotiterplatten-Sunrise Basic tecan) and a comparison of values after the incubation period was attempted. The optical density was measured after an incubation of approximately 24 hours to compare treatment effect between groups.



Picture 4: After the irradiation of Candida albicans in solution in 96 well plates it was incubated on 30°C for about 24h. A1: Control, C1: Just UV, F1: UV + Riboflavin, H1: Just Riboflavin.

In one pilot test the fungi were irradiated on the sabouraud plates. 5µl drops of colonial solution with or without Riboflavin were placed on the agar surface. These drops were dried at room temperature for 15 minutes and then irradiated. This experimental trial failed due to too rapid diffusion of the Riboflavin drops diffused into the sabouraud medium.

Picture 5: On this pilot test the irradiation of Candida albicans took place on the sabouraud agar plate und was incubated for about 48h on 30°C. We made four different plates, from left to right: UV + Riboflavin, just UV, just Riboflavin, Control.

UV-A Irradiation:

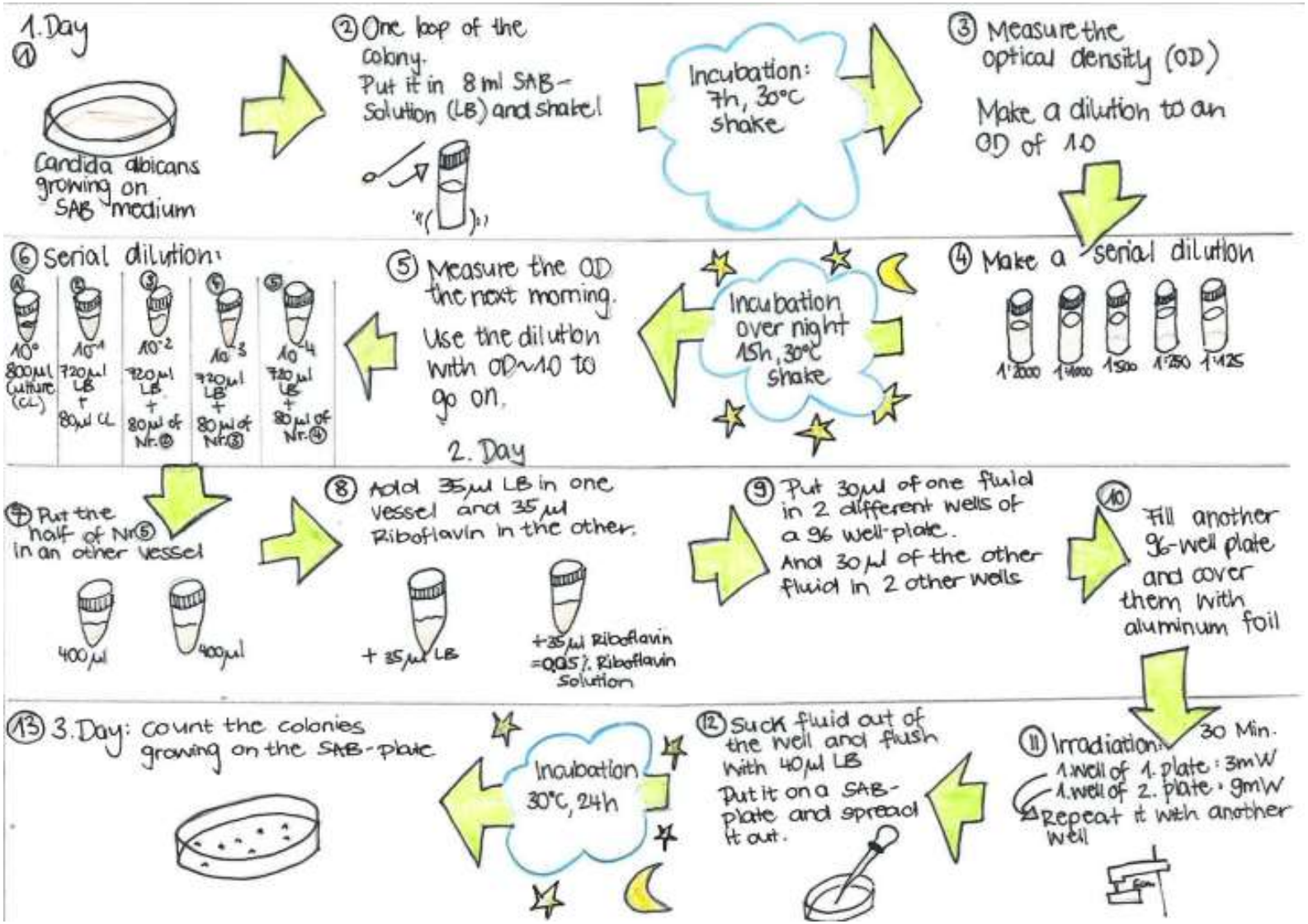
In this experiment two different UV-A lamps were utilized. Both of them have a wavelength of 365 nm. This wavelength correlates to one of the peaks in the absorption spectrum for Riboflavin. The power density of the first lamp is 3mW/cm^2. This results in a total energy delivery of 5.4J/cm^2 after 30 minutes of irradiation. The second lamp has a power density of 9mW/cm^2, which results in a total dose of 16.2J/cm^2 after 30 minutes of irradiation.
The power density output of the two lamps was measured with a UV meter every morning before the experiments were started. The energy output of the 3mV lamp was consistently between 2.7 and 3.3mW/cm^2. The energy output of the 9mV lamp was consistently between 7.0 and 7.75mW/cm^2. The lamp was placed such that the light source was at a distance of 5cm from the table, which corresponds to the focal distance of the light source.

Riboflavin solution:

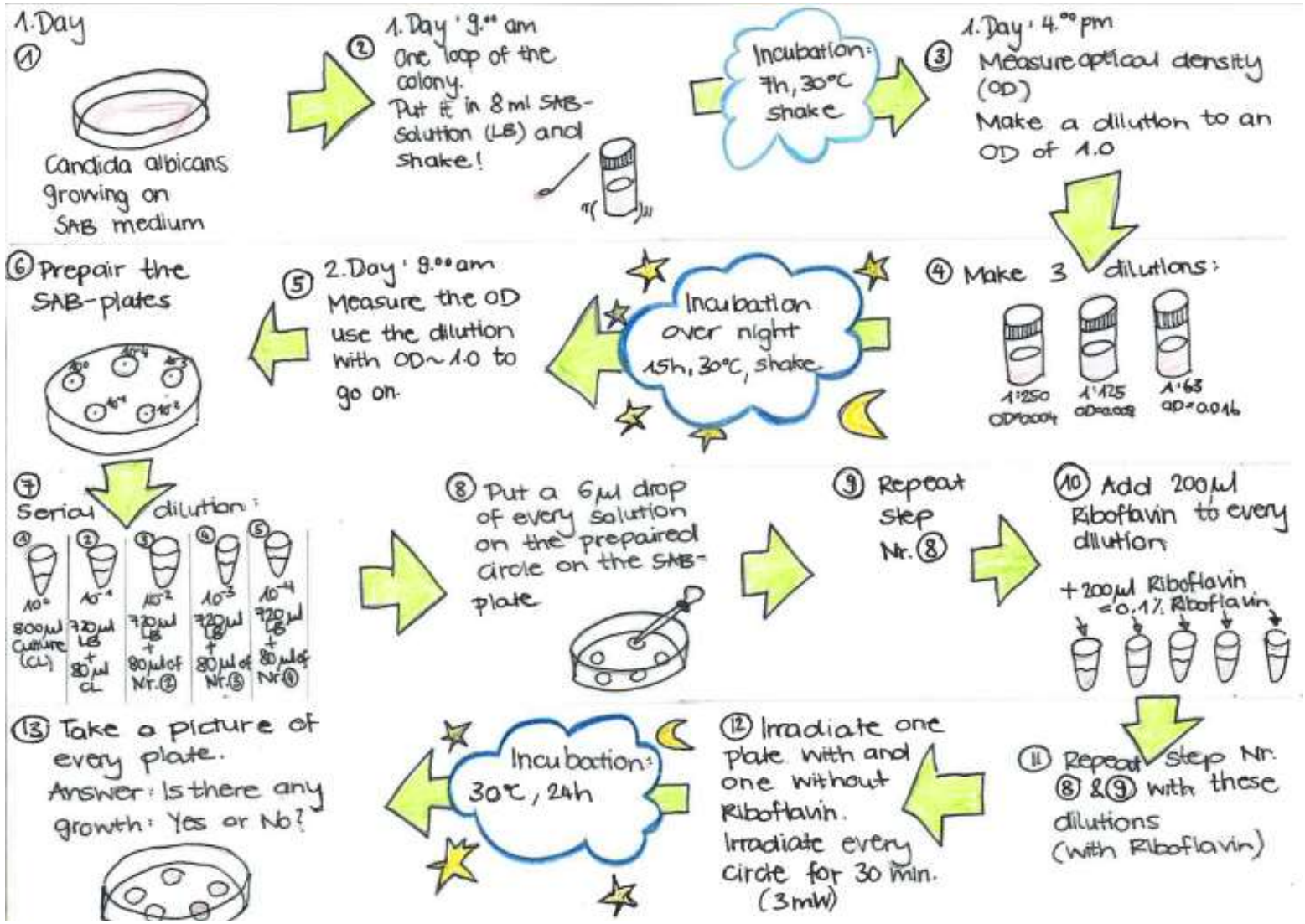
In this experiment a Riboflavin concentration of 0.05% was used. This concentration was achieved by diluting a 0.5% Riboflavin solution (Vitamin B2 Streuli Inj Lös 10 mg/2ml) by adding 35µl 0.5% Riboflavin to 400µl colonial suspension
In most of the other similar studies a Riboflavin concentration of 0.1% was used.[56] In this study a concentration of 0.05% was used because of the higher energy dose and the fact that horses have a thicker cornea than humans and small animals. With the lower concentration of Riboflavin we increase the penetration depth.
A 0.1% Riboflavin concentration was used in various preliminary experiments using both the low and the high-energy protocol. 200µl Riboflavin needs to be mixed with 800µl colonial suspension to create a solution with 0.1% Riboflavin.

Statistical analysis:

In order to assess if the predictors/factors (Riboflavin, UV-A, interaction between Riboflavin and UV-A, total energy dose, isolates) were significantly associated with the response variable (Number of colonies after incubation period) a negative binomial model with random effects for the different trials was utilized. Model selection was based on AIC (Akaike information criterion) with lower AIC values indicating a better model fit. The analysis was performed with the software R[108] version 3.2.0 and the package MASS[109] and nlme[110].



Picture 6: Schematic representation of the final study protocol.



Picture 7: Schematic representation of one of the pilot tests.

Results:

No significant difference in fungicidal effect was observed between treatment groups and untreated controls (no association within the factors could be demonstrated). Even the high energy protocol had no significant effect on the number of colonies after incubation. No difference was observed between isolates either. The boxplot also shows no effect between groups on the survival rate of *Candida albicans* in this experimental system.

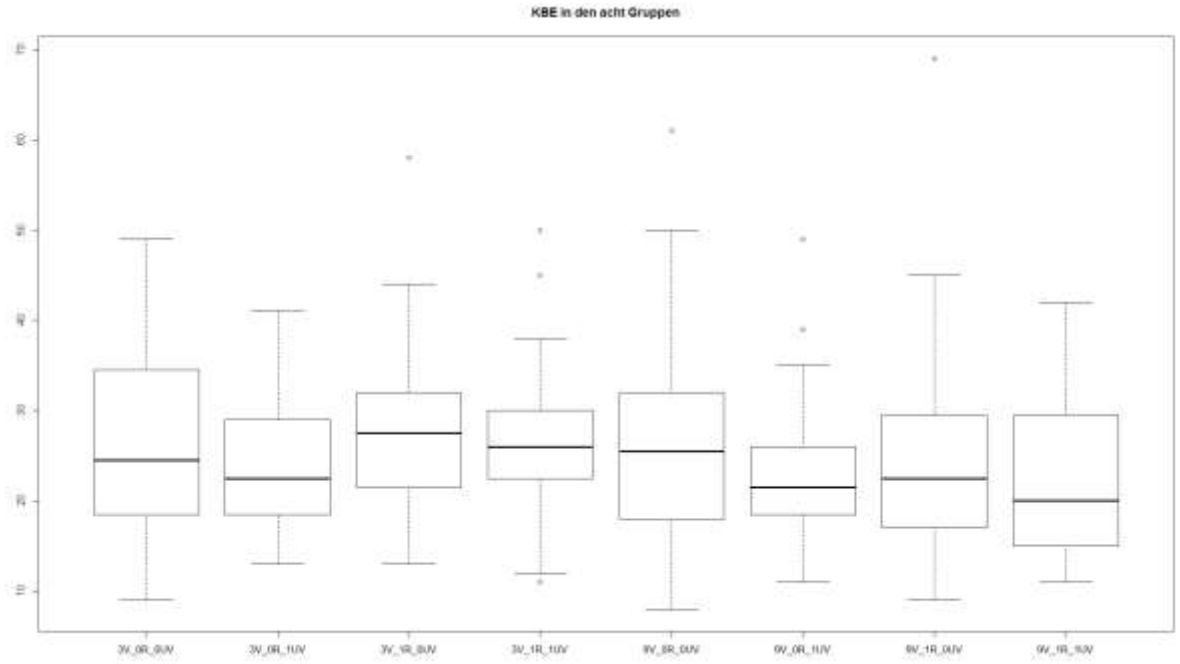


Diagram 1: Boxplots of all the results, the results obtained with the two isolates of *Candida albicans* were combined. Based on the results from the negative binomial models, there was no evidence that any of the factors investigated was significantly (at the level of 0.05) associated with a decreased number of colonies after incubation. Eight groups depicted on the X-axis: with (1) and without (0) Riboflavin, with (1) and without (0) UV-A, 3mW/cm^2 (3V) and 9mW/cm^2 (9V) irradiation protocols used in the trial; y-axis: number of colonies counted on the sabouraud plates after incubation.

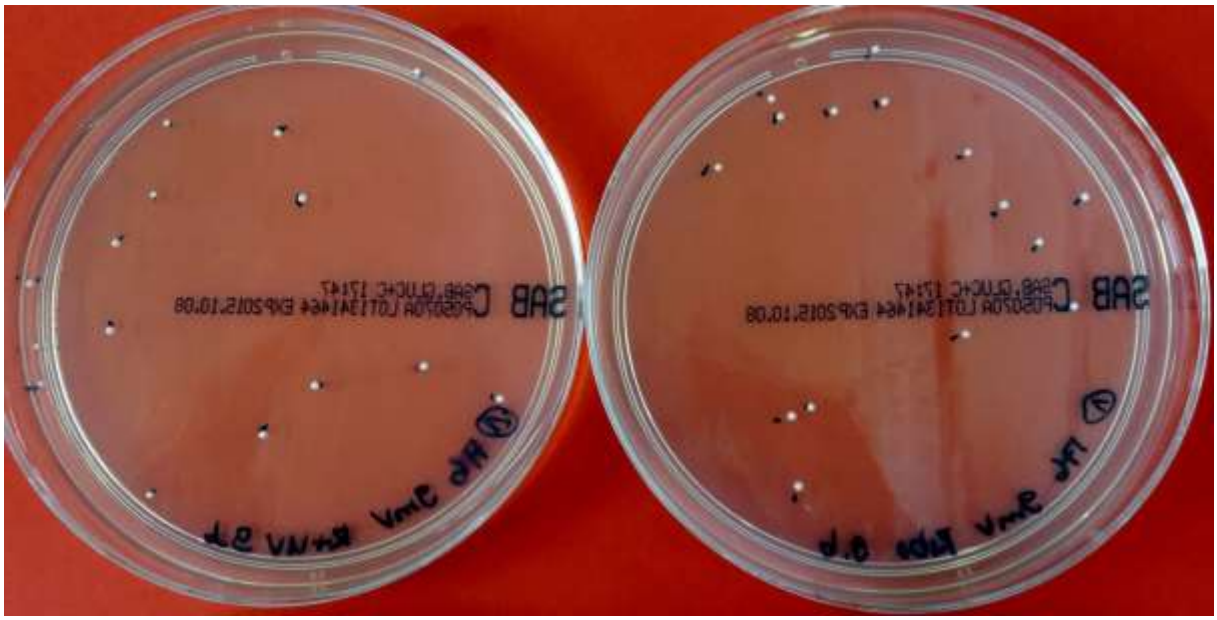
We can rule out small sample size as a possible problem since we included a high number of repeats in this study (28 in total for both isolates).

In some trials it seemed as if the colonies were smaller post UVA + R. But these were just subjective findings. We tried to measure the optical density (tecan reader, Tecan ELISA-Mikrotiterplatten-Sunrise Basic tecan) after irradiation and incubation period to compare the amount of colonies. But even with the measurement there could not be shown any difference (see table 5).

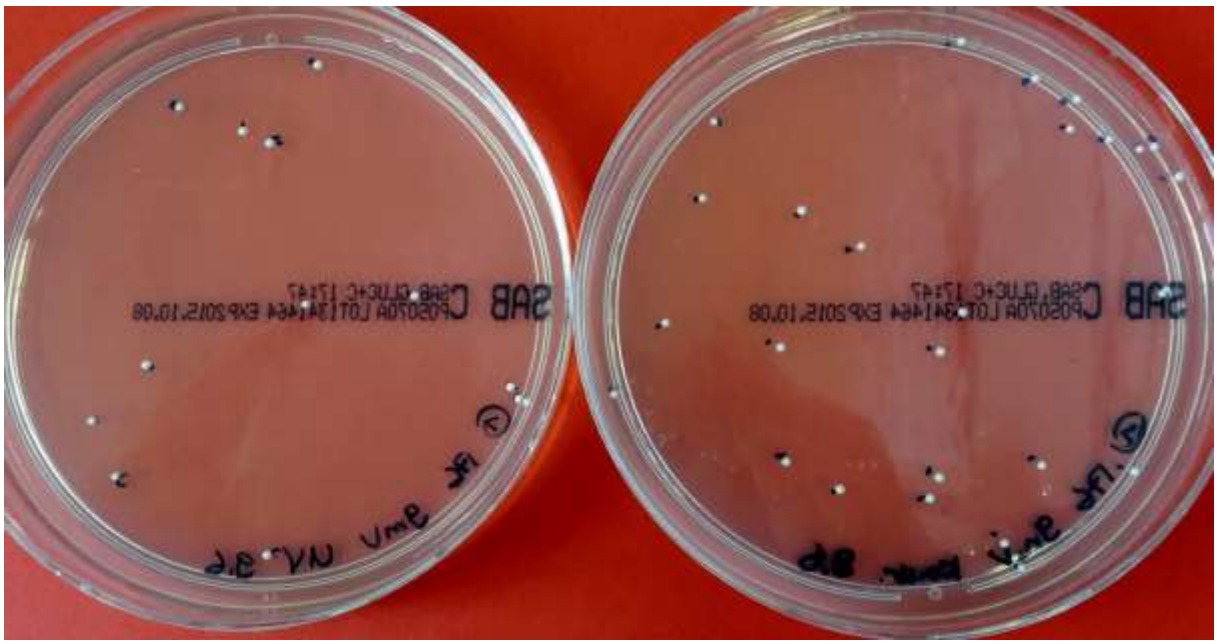
	Just UV	Control	Just Ribo	R + UV
Optical density	1.075	0.969	0.097	0.649
	0.646	0.775	0.077	0.343

Table 5: Measured optical density of the fluid and *Candida albicans* colonies irradiated with UV-A light and then incubated for approximately 24 hours. The optical density was measured with the tecan reader.

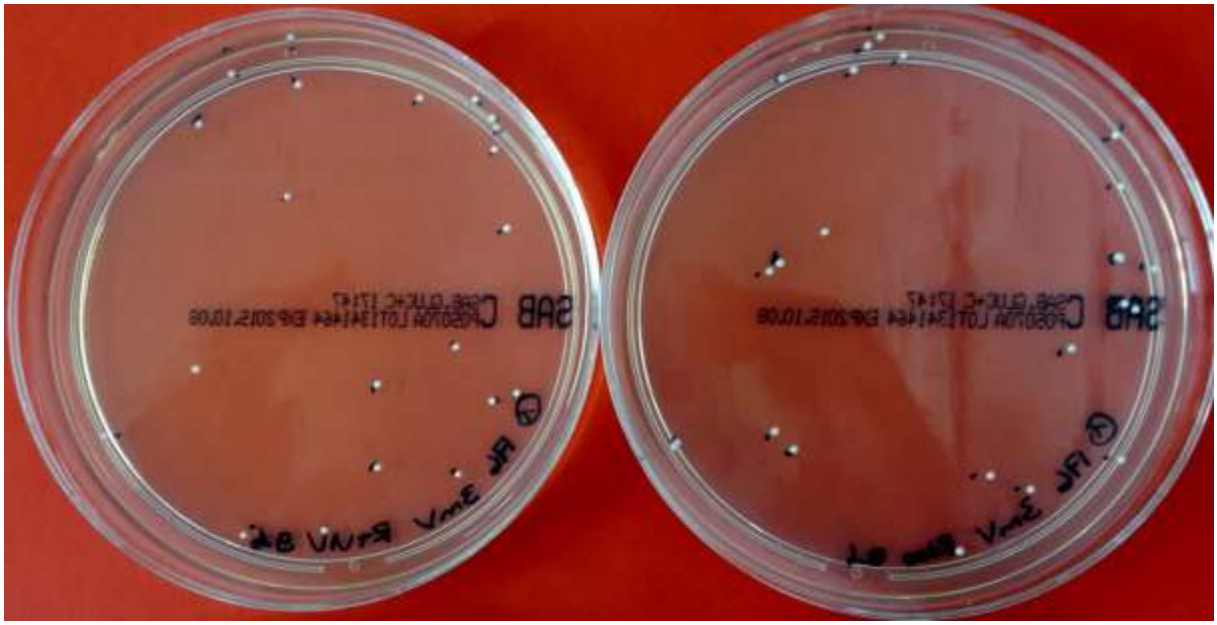
The other preliminary experiments described in the materials and methods section all yielded negative results as well. The experiment in which 0.1% Riboflavin concentration was used with the low and the high-energy delivery protocols showed no difference in number of colonies between the irradiated and the non-irradiated plates. The pilot study in which the fungi were irradiated on the sabouraud plates also demonstrated no visible effect of CXL.



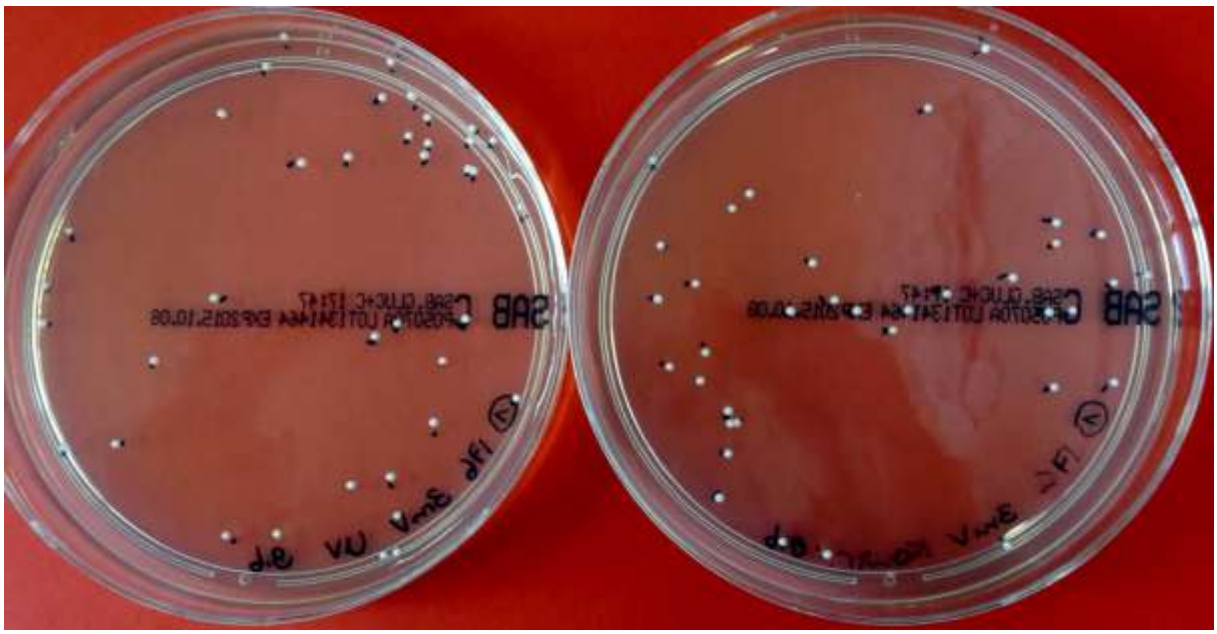
Picture 8: Sabouraud plates after ± 24h incubation at 30°C. Left plate: *Candida albicans* was irradiated with UV-A light + Riboflavin at a power density of 9mW/cm^2. Right plate: *Candida albicans* was saturated with Riboflavin but not UV-A irradiated.



Picture 9: Sabouraud plates after ± 24h incubation on 30°C. Left plate: *Candida albicans* was irradiated with UV-A light at a power density of 9mW/cm^2. Right plate: *Candida albicans* was not saturated with Riboflavin and not UV-A irradiated.



Picture 10: Sabouraud plates after ± 24h incubation on 30°C. . Left plate: *Candida albicans* was irradiated with UV-A light + Riboflavin at a power density of 3mW/cm^2. Right plate: *Candida albicans* was saturated with Riboflavin but not UV-A irradiated



Picture 11: Sabouraud plates after ± 24h incubation on 30°C. Left plate: *Candida albicans* was irradiated with UV-A light at a power density of 3mW/cm^2. Right plate: *Candida albicans* was not saturated with Riboflavin and not UV-A irradiated.

Control 3mV	UV 3mV	Ribo 3mV	R + UV 3mV	Control 9mV	UV 9mV	Ribo 9mV	R + UV 9mV	Isolat
25	20	32	25	36	21	16	36	175
24	29	34	26	17	23	21	15	175
37	26	31	35	22	21	37	33	175
22	20	29	26	22	21	69	35	175
33	22	13	26	17	25	29	16	175
17	27	18	31	30	19	28	11	175
16	21	16	31	29	24	44	20	175
19	15	23	34	35	27	30	30	175
9	16	31	50	27	22	28	42	175
35	19	58	28	19	39	24	36	175
49	28	25	23	27	18	23	11	175
18	18	34	13	32	26	29	20	175
23	32	35	24	13	21	13	17	175
40	22	22	26	19	20	19	14	175
36	37	23	21	24	12	15	15	176
42	33	44	38	41	17	19	29	176
24	18	13	24	33	14	11	19	176
10	15	22	18	61	35	20	15	176
33	41	29	45	15	49	33	21	176
16	29	29	27	19	27	22	20	176
28	14	33	12	21	22	17	21	176
34	35	24	26	50	18	17	16	176
32	22	17	25	8	13	29	15	176
21	13	19	22	16	19	9	14	176
26	28	32	29	32	23	45	34	176
23	23	21	27	11	32	32	24	176
39	27	28	11	32	26	10	23	176
15	34	27	14	29	11	20	25	176

Table 6: Raw data of the 28 repeats. The numbers represent the number of colonies grown on the Sabouraud plates after ± 24h incubation on 30°C.

Discussion:

The most important result of this study is that *Candida albicans* is resistant to CXL. The results show that *Candida albicans* is resistant against standard and high energy delivery (2.5 x standard CXL) protocols. No differences in CFU counts were observed between the CXL treated and control cultures.

With an absence of observable fungicidal CXL effect in vitro, where important variables that influence CXL efficacy can be controlled, we expect the clinical fungicidal effect of CXL in vivo to be variable at best. The thickness of the cornea, presence and density of edema, the presence, density and depth of inflammatory cell infiltrates, ulcer depth, number of fungal organisms, the exact location (especially depth) of the fungi in the cornea, etc. are all important variables influencing CXL efficacy in vivo.

The results of the present in vitro study correspond to the clinical experience with equine keratomycosis and CXL as therapy, where the outcome is not reproducibly successful.[111-115]

A recently published study in human patients describes a higher perforation risk in keratomycotic eyes treated with CXL compared to a medically treated control group.[114] However, this study has to be interpreted with caution because the sample size was small and the affected eyes were treated in a very advanced stage of the disease.

The results of the present study are in line with several similar recently published in vitro studies in which CXL failed to demonstrate any growth reducing effect on *Candida albicans*.[56,87,100,116] At present, only Bing Sun et al. described a growth reducing effect of CXL on *Candida albicans*.[99] Bing Sun et al. also recognized a negative correlation between suspension concentration and the growth inactivation ratio. The experimental protocol used in their study was similar to the one used in our study. In their discussion Bing Sun et al. pointed out that the height of the irradiated liquid columns was a potential weakness in their study since the columns were too high to allow lethal CXL energy delivery to the deeper parts of the fluid columns.

As mentioned in the introduction, several studies demonstrated that the cytotoxic damage after CXL was restricted to the anterior 200[13] - 300µm[14-16] of the corneal stroma. Clinically, all structures behind a 400 µm-thick corneal stroma, including the corneal endothelium, iris, lens epithelium and retina, are deemed to be safe from CXL induced cytotoxic damage. [17,18] One should therefore assume that the same is true for microorganisms behind or beneath a 300µm think 0.1% Riboflavin barrier, especially in the case of a relatively CXL resistant organism like C albicans.

If only a part of the column of C.albicans suspension was actually CXL treated in the study performed by Bing Sun et al., one would expect a more obvious effect using a study protocol using lower liquid columns, as in our study. Also, an increased total energy delivery and lower Riboflavin concentration to increase the penetration depth were used in our study. The absence of CXL induced C. albicans growth suppression presented in this thesis is therefore difficult to correlate to the presence of CXL

induced C. albicans growth suppression obtained by Bing Sun et al..Another difference between the studies is the number of experimental cycles. *Bing sun et al.* performed five experimental cycles while we had 28 cycles in our study.
The liquid column height and the CXL energy penetration depth seem to be a challenging topic in this trial. The major problem is depth limitation of lethal energy delivery, which is limited to 300µm with the standard CXL protocol. This also depends on the Riboflavin concentration with lower Riboflavin concentrations allowing more UV-A energy penetration. Whereas *in vivo* this phenomenon is utilized as protection in an *in vitro* proof of principle study of course the aim is to irradiate the whole sample fluid column to maximize the growth suppressive effect.
To find out the maximal allowable energy level, which could be used in equine corneas to reach the entire corneal stroma more studies regarding the penetration depth should be started in the future.
The main and possibly critical weakness in our study was the creation of a layer of fluid with a uniform thickness of < 300 µm, fluid surface tension was a critical factor.
Another potential reason why this experiment failed to demonstrate CXL induced growth suppression of C. albicans in culture could be the fact that yeasts have pronounced detoxification mechanisms. For example *Candida albicans* can answer to oxidative stress by activating evolutionarily conserved signaling pathways that drive adaptive mechanisms leading to the detoxification of oxidating agents. For example catalase, which detoxifies hydrogen peroxide (H2O2), or thioredoxin and thioredoxin reductase are activated through these pathways. Even the damage caused by the ROS is going to be repaired.[117-119] Resistance to oxygen radicals was demonstrated in filamentous fungi (*Cercospora*).[120] Such mechanisms could explain the difference between a lack of CXL induced growth inhibition of *Candida albicans*, and > 90% growth reduction in bacterial cultures.[121]
Biofilm formation can also lead to an increased resistance against antimicrobial drugs and inhibits drug diffusion to the cells reducing the concentration of the drug reaching the cells in the biofilm. Such factors may also decrease CXL efficacy by impairing Riboflavin diffusion and uptake.[122] Although *Candida albicans* is able to form biofilms on surfaces, in our case biofilm formation is unlikely because the fungi were always kept in suspension during the pretreatment and irradiation phases of the experiments.[120,123]

Conclusion:

Standard and high-energy delivery CXL protocols did not suppress the growth of *Candida albicans* under the experimental conditions presented in this thesis.
Based on the previous literature and the current experimental results CXL therefore seems to be a promising therapy for bacterial infections in the cornea but not necessarily for keratomycosis.
But even without a direct antifungal effect CXL may be a good option as an addition to antifungal drug therapy due to the CXL induced effects of biomechanical stiffening and biochemical stabilization of the remaining cornea.

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